Temperature Dependence of the Inhibitory Effects of Orthovanadate on Shortening Velocity in Fast Skeletal Muscle

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ABSTRACT We have investigated the effects of the orthophosphate (P_i) analog orthovanadate (V_i) on maximum shortening velocity (V_{max}) in activated, chemically skinned, vertebrate skeletal muscle fibers. Using new "temperature-jump" protocols, reproducible data can be obtained from activated fibers at high temperatures, and we have examined the effect of increased [V_i] on V_{max} for temperatures in the range 5–30°C. We find that for temperatures ≤20°C, increasing [V_i] inhibits V_{max}; for temperatures ≥25°C, increasing [V_i] does not inhibit V_{max}. Attached cross-bridges bound to V_i are thought to be an analog of the weakly bound actin·myosin·ADP·P_i state. The data suggest that the weakly bound V_i state can inhibit velocity at low temperature, but not at high temperature, with the transition occurring over a narrow temperature range of <5°C. This suggests a highly cooperative interaction. The data also define a Q_{10} for V_{max} of 2.1 for chemically skinned rabbit psoas fibers over the temperature range of 5–30°C.

INTRODUCTION

Muscle contraction results from an interaction between myosin cross-bridges and binding sites on adjacent actin filaments. After binding, a number of lines of evidence from biochemical and mechanical studies suggest that orthophosphate (P_i) release from the low force, weakly attached A·M·ADP·P_i state (A = actin, M = myosin) is involved in the conformational change(s) necessary for the production of active tension and shortening (reviewed in Cooke, 1986; Goldman, 1987; Hibberd and Trentham, 1986). This apparently pivotal role of P_i release in the actomyosin chemomechanical cycle has received additional support from the x-ray crystal solution of myosin subfragment-1 (S1) (Rayment et al., 1993a) and the "docking" of this crystal structure to the derived structure of F-actin (Rayment et al., 1993b). When nucleotide is modeled into the S1 structure, the γ-phosphate is poised at a hinge formed by a large cleft in the protein structure that extends from the actin binding site to the substrate binding pocket. P_i release is proposed to be involved in the opening and closing of the cleft, mediating communication between the site that binds actin and that which binds nucleotide.

To better understand the relationship between P_i release and actomyosin chemomechanics, numerous studies have investigated the effects of increasing [P_i] on actively contracting, chemically skinned, fast skeletal muscle fibers. Elevated [P_i] increases the fraction of cross-bridges in the weakly attached, pre-powerstroke A·M·ADP·P_i state. Mechanically, this depresses isometric tension and mechanical stiffness, while leaving maximum shortening velocity (V_{max}) unaffected (Altringham and Johnston, 1985; Brozovich et al., 1988; Chase and Kushmerick, 1988; Cooke and Pate, 1985; Hibberd et al., 1985; Martyn and Gordon, 1992; Millar and Homsher, 1990; Walker and Moss, 1992; Dantzig et al., 1992).

An alternative approach to understanding the properties of the pre-powerstroke states has been through the use of P_i analogs. Phosphate analogs such as orthovanadate (V_i), or the metallofluoride complexes of aluminum, bind to an A·M·ADP state in the active cycle. The resulting M·ADP·analog state binds to actin in fibers (Chase et al., 1993; Dantzig and Goldman, 1985; Wilson et al., 1991), stabilizing the cross-bridges in a state thought to resemble the pre-powerstroke state, and increasing the relative fraction of the cross-bridge population that this state represents. A significant advantage of these analogs is that they bind much more tightly than P_i, and thus significantly less ligand is required to induce equivalent mechanical perturbations. Mechanical studies indicate that, like P_i, increasing concentrations of these P_i analogs decrease isometric tension, stiffness, and hydrolysis rates in fast skeletal fibers (Chase et al., 1993; Dantzig and Goldman, 1985; Wilson et al., 1990). Observations of the effects of these analogs on shortening velocity remain disparate, however.

Wilson et al. (1990) reported that V_{max} was unaffected by V_i concentrations that reduced isometric tension to less than 5% of control values. Contrarily, Chase et al. (1988) have found that V_{max} decreases roughly linearly with tension as the [V_i] increases. A significant difference in the protocols employed by these two investigations was the temperature at which the experiments were performed. Chase et al. worked in the temperature range 10–13°C; Wilson et al. worked at a higher temperature of 25°C. Given these reported differences, we have more extensively investigated the effects of

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V_i on shortening velocity as a function of temperature over the range 5–30°C.

We find that the effect of V_i on V_{max} displays a marked temperature dependence. For temperatures ≥25°C, we find that addition of 1 mM V_i does not inhibit shortening velocity. For temperatures ≤20°C, 1 mM V_i reduces V_{max} to approximately 50% of control values. There is a sharp transition region between 20 and 25°C. Thus, our observations provide a resolution of the previously disparate reports on the effects of V_i on V_{max}. They also show that the effect of V_i on velocity shifts dramatically over a narrow range of temperatures.

Chemically skinned fibers are increasingly unstable as the experimental temperature is raised. This has previously limited their use to lower temperature regimes. To extend our observations on V_i to temperatures as high as 30°C, new "temperature-jump" experimental protocols have been developed that allow us to obtain reproducible data from fibers activated at higher temperatures. Both the mechanical parameters and the fiber sarcomere laser light diffraction patterns are stable for sufficient periods of time for data collection. These protocols are described here, and allow us to define a Q_{10} for V_{max} of approximately 2 over the temperature range 5–30°C.

MATERIALS AND METHODS

Rabbit psoas muscle was harvested and chemically skinned as described previously (Cooke et al., 1988). Single fibers were dissected and mounted between a solid state force transducer (Sensonor model 801, Horten, Norway) and a rapid motor (General Scanning) for changing muscle length during isotonic release experiments. Mounting techniques and the experimental apparatus have been described previously (Cooke et al., 1988) except for the modifications detailed below that were necessary for experiments at higher temperatures.

The solution for activating fibers contained 8 mM magnesium acetate, 100 mM *N*-tris[hydroxymethyl]-methyl-2-aminoethane sulfonic acid (TES), 1 mM EGTA, 1.1 mM calcium acetate, 5 mM K_2HPO_4, 20 mM creatine phosphate, 2 mg ml^{-1} creatine phosphokinase, with variable amounts of Na_2ATP and potassium acetate (approximately 6 mM and 0.08 M, respectively), maintaining a constant, physiological, 5 mM MgATP concentration, and ionic strength of 200 mM, pH 7.0, as temperature varied between 5 and 30°C. The composition, pH, and ionic strength of the solutions used during fiber activation were determined from standard binding constants using a FORTRAN computer program.

For experiments involving addition of vanadate to experimental buffers, stock solutions of sodium metavanadate ($NaVO_3$) were made at 100 mM, pH 10. The high pH reduced the extent of polymerization during storage. To hydrolyze any polymerized vanadate species, the stock solution was always boiled before being used. This procedure eliminated any yellow or orange color caused by polymerization (Goodno, 1982; Penningroth, 1986; Rubinson, 1981). The high concentration of pH buffer in the activating solution (100 mM TES) was present to compensate for pH changes when V_i was added to the fiber activating solutions. When V_i was added to fiber activating solutions in concentrations up to 5 mM from stock V_i solutions at pH 10, increases of not more than 0.1 pH unit were observed as recorded by direct measurement. In the present experiments, 2 mM added V_i was the highest concentration employed. The concentrations of V_i species were not included in the calculation of ionic strength.

For isotonic releases, the fiber was allowed to shorten against a preset fraction of isometric tension (P_o) for 40 msec. The fiber was then rapidly slacked by 1 mm, the true zero voltage on the force transducer determined, and the fiber rapidly stretched to the original mounted length. A least-squares, linear fit as a function of time of the position of the motor arm to which the fiber was attached was determined over the period 10–40 msec after release. The slope was taken as the shortening velocity. The accumulated force-velocity data for a given experimental condition were fit to the Hill equation (Hill, 1938), with the extrapolated value of velocity at zero tension taken as V_{max}. The feedback loop between the force transducer and the motor was under computer control and operated at a frequency of 4 kHz. The resonant frequency of the force transducer was 4 kHz. Additional details of the experimental apparatus and fitting protocols are described in Cooke et al. (1988).

It is well known that chemically skinned fibers are not as mechanically stable as living fibers. This has restricted their use to lower temperatures where fiber activation and data collection can be accomplished before mechanical reproducibility deteriorates. The following protocol has been employed to circumvent this problem at higher temperatures. A fiber was initially mounted in a well containing a Ca^{2+}-free relaxing buffer (previously described experimental buffer with Ca^{2+} omitted) with temperature maintained at <2°C. Two minutes were allowed for equilibration of exogeneous creatine phosphokinase across the fiber. The fiber was then activated by addition of Ca^{2+} to the well in which the fiber rested. At this low temperature, fibers produced little force and were extremely stable. Constant laser light diffraction patterns could be maintained for many minutes. The pH, buffer constituents, and ionic strength of the experimental buffer in the 2°C well were adjusted as appropriate for the higher temperature at which data were desired, because these change (particularly pH) with temperature. This required the pH of the 2°C buffer to be 7.4 (as determined by direct measurement) to obtain a buffer pH of 7.0 at 30°C. The fiber was then rapidly (≤1.0 s) transferred to a well containing an identical experimental buffer maintained at the temperature for which data were desired. The temperature equilibration across a 75-μm diameter fiber will be on the order of 10 ms. Reequilibration of pH and ionic strength is virtually instantaneous with respect to temperature change, and as is evident from Fig. 1, force generation is 90% complete in approximately 100 ms. This is comparable to that observed in living fibers at in vivo temperatures (Close, 1972). Thus, with the equilibrations required for fiber activation occurring an order of magnitude more rapidly than the chemomechanical processes involved, our protocol circumvents the relatively long time (approx. 1–2 s) for diffusive equilibration of Ca^{2+} across the fiber, the problems of temporal and spatial nonuniformity of activation, and the lack of mechanical reproducibility of

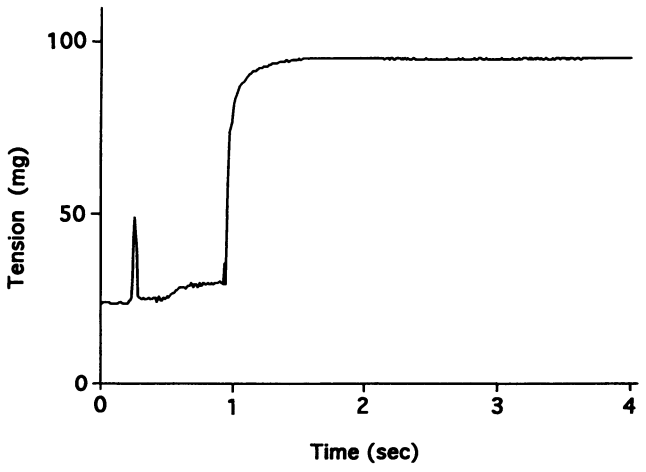

FIGURE 1 The time course of the isometric tension of a single psoas fiber after transfer from an activation buffer at 2°C to an activation buffer at 30°C. The fiber was fully activated, pCa ~ 4.5, at both temperatures. The initial tension spike results from passage through the surface meniscus of the low temperature buffer. Temperature and tension rise slightly when the fiber arrives over the high temperature well. After submersion in the high temperature buffer, tension rises rapidly to a new steady-state level. Maximum isometric force (0.31 N/mm^2) is obtained in approximately 100 ms. Isometric tension at 2°C was .077 N/mm^2, and the tension of the relaxed fiber was 0.002 N/mm^2. Fiber diameter = 62 μm, length = 3.1 mm.

the fiber. One problem that arose occasionally was a modest warming of the fiber during transit between wells. Most experiments reported here were done during the summer months in a laboratory at UCSF, chilled by Pacific Ocean fog, and warming was not a problem. On the rare warm days it was determined that the fiber tension would begin to rise during the transfer between experimental wells. To circumvent this problem, a container of dry ice with a grating in the bottom was placed above the apparatus during the transfer. The descending CO_2 fog replaced the natural refrigerant.

With the experimental apparatus under computer control, repeated isotonic releases to varying preset fractions of isometric tension (P_o) could be rapidly accumulated. Fig. 2, *a* and *b* show results from two such isotonic releases performed at 30°C. Panel *a* gives the position of the motor arm to which the fiber is attached as a function of time, along with least-squares, linear fits whose slopes give the velocities of shortening. Release tensions are given in panel *b*, with the higher release tension corresponding to the lower velocity. We have previously shown that the concentrations of creatine phosphate and creatine phosphokinase employed in the study are sufficient to buffer internal nucleotide concentrations at low temperatures (Cooke and Bialek, 1979). To ensure adequate substrate buffering at the higher temperatures, additional releases were done with the creatine phosphate and/or creatine phosphokinase concentration(s) doubled. No change in velocity was observed at 30°C, indicating adequate buffering at the higher temperature as well. We note, however, that unless a minimum 1 min equilibration time in the <2°C buffer was allowed for creatine phosphokinase to equilibrate within the fiber, reduced velocities were obtained at the higher temperature. Fig. 2, *c* and *d* give similar isotonic release data at 30°C, with 1 mM V_i added to the experimental buffer. Fig. 3 (*top*) shows a laser light diffraction pattern from the fiber in the relaxed state for the fiber in Fig. 2, *a* and *b* (0 mM V_i). Fig. 3 (*bottom*) shows the diffraction pattern after the isotonic releases. Although the diffraction pattern under activating conditions is weaker than that from the relaxed state, it is nonetheless clear that stable sarcomere diffraction patterns were maintained during the time course of data collection for even those conditions that yielded our highest isometric tensions. Isotonic releases were performed in sets of three spaced 2 s apart. In each set after the first, one release duplicated a release tension from the first set. A change in velocity of >10% resulted in termination of the experiment.

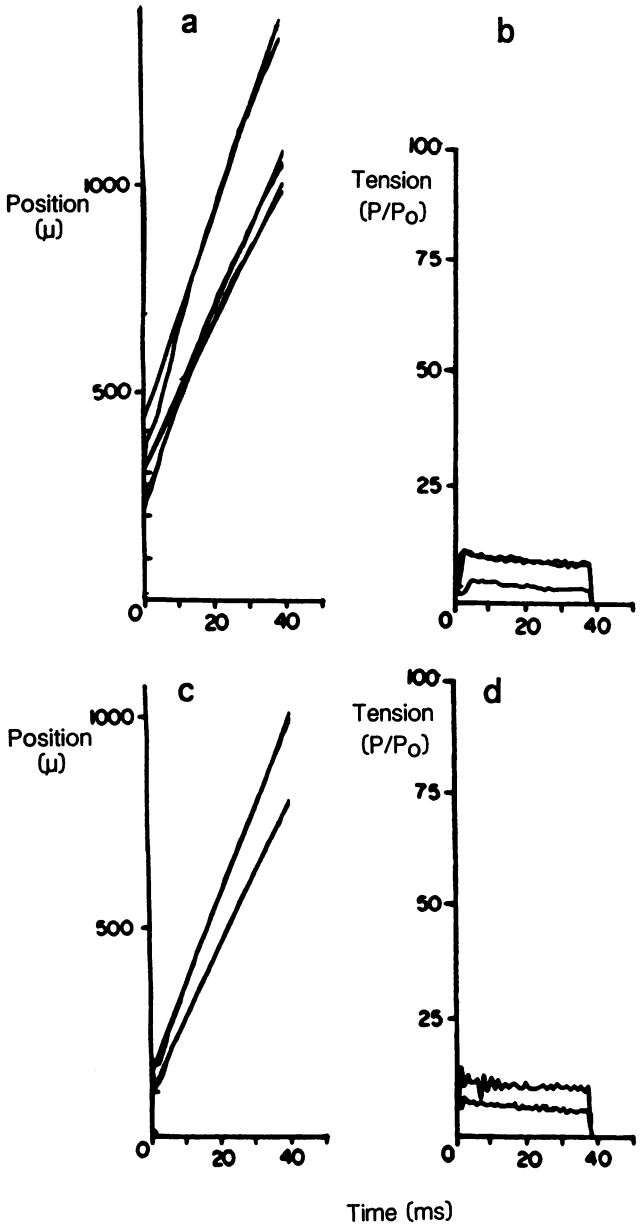

FIGURE 2 Isotonic release data at 30°C. (*a*) Position of the motor arm to which the fiber was attached as a function of time. The slope of the least squares, linear fit to the position data is taken as the shortening velocity. (*b*) Release tensions as a function of time. (*c*) and (*d*) Position and release tension data in the presence of 1 mM added V_i. Fiber length = 4.1 mm (*a, b*), 4.2 mm (*c, d*). For the traces shown above (*a, b*) the velocities are 4.12, 5.75, 4.54 lengths/s for a sequence of release tensions to 10, 3, and 8% of P_o; and for the traces shown below the velocities are 4.65 and 5.64 lengths/s for a sequence of release tensions of 11 and 6% of P_o. The higher velocities correspond to the lower release tensions. Care was taken to add a saturating amount of Ca to ensure complete fiber activation under all conditions.

RESULTS

Fig. 4 shows the accumulated force-velocity data and Hill fits for isotonic releases at 15°C (*lower two fits*) and 30°C (*upper two fits*). The lower pair shows a distinct temperature dependence of the effect of V_i on V_{max}. At 15°C, addition of 1 mM V_i decreases V_{max} by 50% (from 2.78 ± 0.11 to 1.41 ± 0.10 l/s; errors are 95% confidence limits on the extrapolated value). At 30°C, V_{max} = 6.21 ± 0.13 l/s without V_i (*open circles*) and 6.53 ± 0.21 l/s with 1 mM V_i (*open triangles*). Thus, we do not see an effect of V_i on V_{max} at sufficiently high temperature. The data at 30°C do indicate a slightly higher curvature for the force-velocity data in the presence of 1 mM V_i, however.

Similar force-velocity data were accumulated over the temperature range 5–30°C. Fig. 5 gives the relative shortening velocity as determined from the Hill fits (V_{max} with 1 mM V_i/V_{max} without added V_i) as a function of temperature. Consistent with our previous observations, for low temperatures (≤20°C), V_i produces a decrease in V_{max}. For high temperatures (≥25°C), V_i has no effect on V_{max}. There is a rather sharp transition region spanning at most 3–5°C between the two modes of behavior. The effect of $[V_i]$ on isometric tension was similar at both high (25°C) and low (5°C) temperatures. In both cases the tension decreased as V_i was raised above 1–5 μM and was inhibited by 90% at about 1 mM.

Additional control experiments were also performed. As noted previously, isometric tension decreases with increasing $[V_i]$, and Fig. 5 shows the results from experiments at a

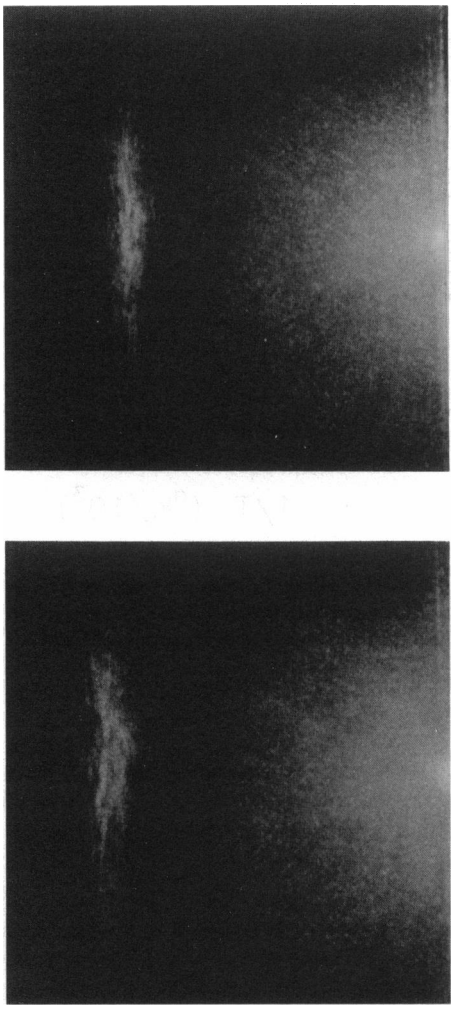

FIGURE 3 Diffraction patterns for a mounted, chemically skinned fiber in the relaxed state (*top*) and in the active state after three isotonic releases (*bottom*). The sarcomere length in *a* was 2.63 microns at full activation at 30°C. Upon activation, the sarcomere length decreased to 2.40 microns, as can be seen from the small shift in the position of the diffraction line (increase in spacing = 9.4%)

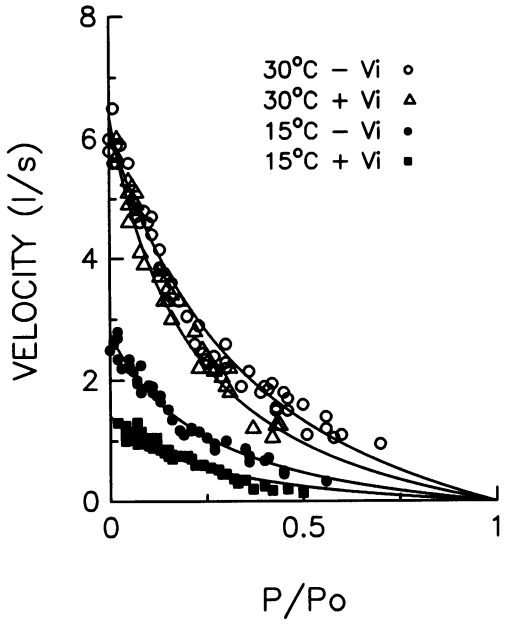

FIGURE 4 Accumulated shortening velocity data as a function of release tensions at 15°C (*closed circles*, 0 V_i; *closed squares*, 1 mM V_i) and 30°C (*open circles*, 0 V_i; *open triangles* 1 mM V_i). Solid lines are hyperbolic Hill fits to the data. As is evident, V_i has an effect on V_{max} at 15°C but not at 30°C.

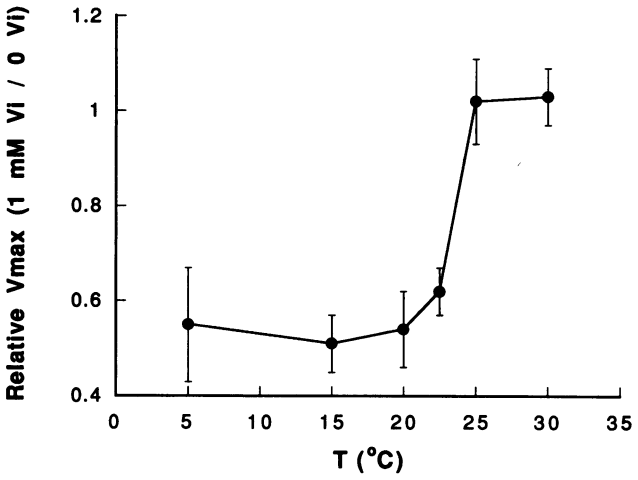

FIGURE 5 Relative maximum shortening velocity (V_{max} with 1 mM added V_i/V_{max} without added V_i) as a function of temperature. For lower temperatures, 1 mM V_i inhibits shortening velocity. For higher temperatures 1 mM V_i does not inhibit shortening velocity. There is a sharp transition between the two regions. Error bars represent upper and lower 95% confidence intervals on V_{max} in the presence of 1 mM V_i divided by lower and upper confidence intervals, respectively, in the absence of added V_i.

single [V_i], 1 mM. Fig. 6 shows the relative value of extrapolated V_{max} obtained with variable concentrations of V_i (10 μM–1 mM) added to fibers at 25°C. The solid line is a least-squares, linear fit to the data. The slope of the linear fit is 0.02 ± 0.05 (SD), implying that no statistically significant variation in velocity is detected over the range of V_i concentrations examined. Chase et al. (1993) examined unloaded shortening velocity (V_{us}) as P_o was decreased by increasing the concentrations of P_i, V_i, or aluminum fluoride complex at a lower temperature, 10–13°C. We wish to compare their results to the present experiments. Fig. 7 shows the results from protocols mimicking those employed by Chase et al. A fiber was initially activated in either the absence or presence of V_i (10 μM–1 mM), isometric tension was recorded, and a low tension isotonic release to 1 or 10% of P_o was performed (V_1 or V_{10}, respectively). The fiber was then rapidly returned to a relaxing buffer, and after 2 min, was reactivated under the alternative (with respect to the presence or absence of V_i) conditions. Isometric tension was again recorded and V_1 or V_{10} releases were performed. Fig. 7 gives the ratios of shortening velocities as a function of relative tension at 5°C (*open circles*) and 15°C (*open triangles*). Accepting the ratios of V_1 or V_{10} in the presence/absence of V_i as an approximation to the ratios of the values for V_{us}, the crucial observation is the similarity between Fig. 7 and the ratios for V_{us} determined by Chase et al. (their Fig. 7 *A*).

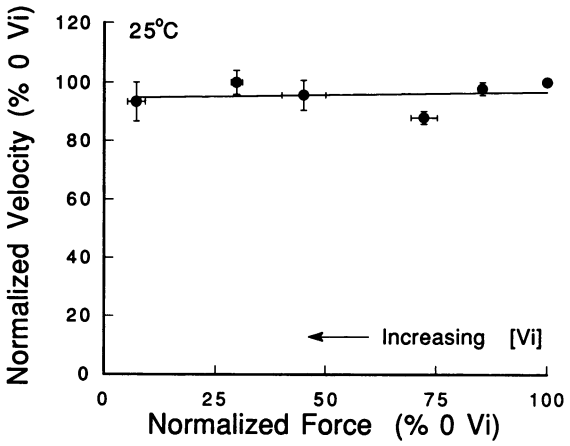

FIGURE 6 Maximum shortening velocity as isometric tension is varied by addition of V_i (10 μM–1 mM) to experimental buffers for fibers contracting at 25°C. Horizontal axis gives isometric tension normalized to that obtained in the absence of added V_i. Lower normalized tensions correspond to higher $[V_i]$. Vertical axis gives the extrapolated V_{max} from Hill fits relative to that obtained in the absence of added V_i. Least-squares linear fit has a slope that is not statistically different from 0. Error bars for tension are SEM; error bars for V_{max} are 95% confidence limits. A minimum of eight fibers were used for each data point.

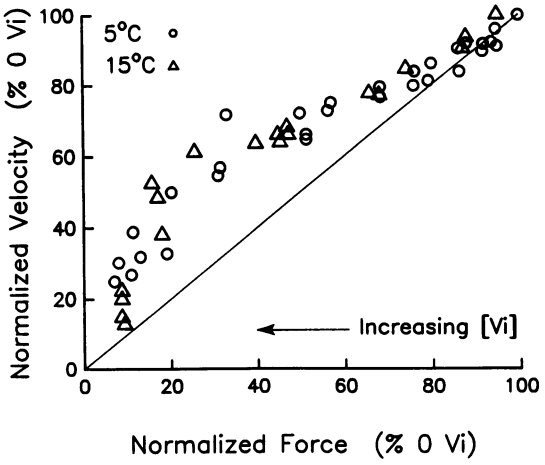

FIGURE 7 Shortening velocity at varying $[V_i]$ (10 μM–1 mM) normalized with respect to that obtained in the absence of V_i versus normalized force for 5°C (*open circles*), 15°C (*open triangles*). Values given as percents. Data use shortening velocities at 1 and 10% P_o as approximations to V_{max} as discussed in Results. The straight line connecting the origin with the 0 V_i control value is given for reference. Data are similar to Fig. 7*A* of Chase et al. (1993) and show a decrease in velocity with increasing $[V_i]$.

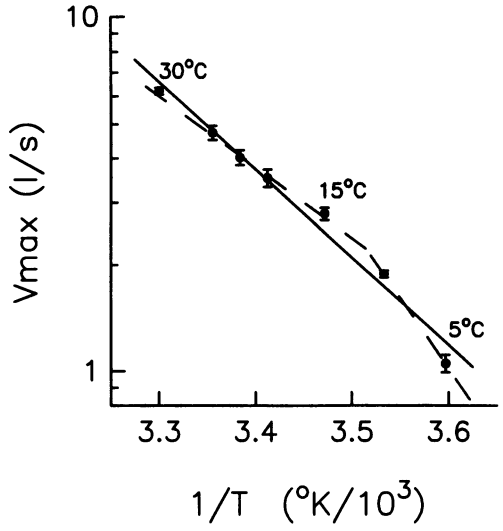

FIGURE 8 Arrhenius plot of V_{max} (log scale) versus 1/temperature (0 K). The data define a Q_{10} for chemically skinned rabbit psoas fibers of 2.1 over the temperature range 5–30°C. Solid line is least-squares fit to all data. Data display a downward concavity at lower temperatures (right side of figure). The dashed lines show that the data is better fit in a piecewise linear fashion.

Shortening velocity is known to increase as temperature increases in living fibers with a Q_{10} (multiplicative increase for a 10°C increase in temperature) of approximately 2 (reviewed in Woledge et al., 1985). The "temperature-jump" protocols employed here allow us to examine the temperature dependence of V_{max} for chemically skinned, fast skeletal muscle fibers over a much larger range of temperature variation than has previously been feasible. Fig. 8 gives an Arrhenius plot of V_{max} versus 1/temperature (K) for data accumulated over the range 5–30°C. The observed values of V_{max} at the two extremes of temperature define a Q_{10} of 2.1. The Arrhenius plot is well fit by a single, least-squares, linear fit (*solid line*, $r^2 = 0.989$). Assuming that V_{max} is governed by a single chemical interaction, the slope of the linear fit defines an activation energy of 48 kJ mol^{-1}. This value is comparable to those that are obtained from measurements of the temperature dependence of shortening velocity in living rat e.d.l. muscle (10–35°C, Ranatunga, 1984), and for type 2 fibers from *Xenopus laevis* (10–20°C, Lannergren et al., 1982), but approximately 25% less than that determined by Stein et al. (1982) for rat e.d.l. and soleus muscles (8–38°C). Our value for Q_{10} is also comparable to that obtained for skinned, fast-twitch fibers from the bulltrout (Johnston and Sidell, 1984), but approximately 30% greater than observed for fast-twitch fibers from a different teleost, the striped bass (Moerland and Sidell, 1986). We note that there is a downward concavity in the present data for temperatures <15°C (lower temperatures to right of Fig. 8), and a better fit is obtained using a piecewise linear approximation (*dashed lines*, $r^2 = 0.996$). The slopes of the two linear fits differ by a factor of 1.7. Similar deviation from linearity has been noted previously (Lannergren et al., 1982; Ranatunga, 1984; Johnston and Sidell, 1984). Although velocity as a function of temperature is the primary thrust of this study, we note that in the absence of added V_i, P_o rose from 0.15 ± 0.01 N/mm² (mean ± SE, 14 observations) at 10°C to 0.29 ± 0.01 N/mm² (17 observations) at 30°C. The value at 10°C is virtually identical to that previously determined in our laboratory at a comparable 5 mM P_i concentration (Pate and Cooke, 1989). The values at 10 and 30°C define a Q_{10} of 1.39 for P_o over the temperature range considered. This value for Q_{10} is almost identical to the value of 1.41 (4–22°C), observed previously by Godt and Lindley (1982) for skinned frog semitendinosus muscle. Previous investigations with living fibers

have also shown that the Q_{10} for P_o is less than that for V_{max}, with our value of 1.39 at the upper end of the observed values (reviewed in Table 2.III; Woledge et al., 1985). Our continued agreement with the data from living fibers provides additional confidence in our experimental protocol.

DISCUSSION

We have investigated the effects of increasing concentrations of the orthophosphate analog orthovanadate on shortening velocity in fast skeletal muscle. This study was undertaken initially to explore possible explanations for two previous investigations that provided potentially conflicting results and that were conducted at different experimental temperatures (no inhibition, 25°C: Wilson et al., 1990; inhibition, 10–13°C: Chase et al., 1993). Hence, we have investigated the effects of V_i on V_{max} as a function of temperature. We find that for temperatures $\geq$25°C, V_{max} is unaffected by increasing $[V_i]$; for temperatures $\leq$20°C, V_{max} is inhibited by increasing $[V_i]$. Thus, our present results are in agreement with both Wilson et al. (1990) and Chase et al. (1993), with the variation in experimental temperature being the culprit in the previously reported differences. However, our studies uncovered an unexpected phenomenon, the presence of an extremely narrow transition zone between the two modes of behavior.

Fiber mechanics measured at high temperatures

The present studies have required the development of new experimental protocols to measure reproducibly isotonic shortening velocities at higher temperatures. It is important to assess the validity of data obtained from our new protocols, which were required to overcome the widely observed, increasing mechanical instability of chemically skinned muscle preparations with increasing temperature. We shall then consider the potential molecular mechanisms involved.

As discussed in Materials and Methods, our solution to the instability problem has been to activate initially the preparation at a low temperature (<2°C) where glycerinated fibers produce low values of force and are exceptionally stable. After diffusive equilibration of the various buffer constituents is achieved, the fiber is then transferred to a buffer maintained at the higher temperature at which data are desired. Temperature equilibration (in contrast to diffusive equilibration of ionic species) and the initiation of a new level of tension generation are uniform and virtually instantaneous across the fiber. This exceptionally rapid "activation" at the higher temperature appears to be the crucial factor. All attempts by ourselves to activate glycerinated fibers at high temperatures via protocols requiring diffusive equilibration have proven uniformly unsatisfactory. As we have demonstrated, with the temperature-jump protocols stable laser light diffraction patterns can be maintained for extended periods of time, during which reproducible isotonic release data can be obtained at even the highest temperatures considered.

Our method differs from other temperature-jump protocols previously employed. Goldman et al. (1987) used laser light illumination and were able to rapidly elevate temperatures, but only by a relatively modest <10°C increase. Bershitsky and Tsaturyan (1992) employed brief, alternating current pulses through fibers and were able to jump temperatures from 5–9 to 40°C. This jump is in the the range reported here; however, a stable elevated temperature could not be maintained. Cooling occurred within 150 ms of the temperature-jump, significantly limiting the period for data acquisition. The protocols employed here appear to solve the limited temperature range of the laser jump method and the temperature stability limitations of the electric current pulse technique.

To assess data quality, it is useful to compare the results obtained at high temperatures to those obtained at low temperatures, and where possible to compare the present results with data previously published from this laboratory. At 10°C, the present experiments yielded an extrapolated V_{max} of 1.88 $\pm$ 0.05 l/s (Fig. 8). This value is only a little greater than our previously reported values of 1.63 $\pm$ 0.05 and 1.76 $\pm$ 0.03 l/s (Cooke et al., 1988). Thus, the temperature-jump protocol may potentially be giving marginally greater velocities at even the lower temperature. No direct comparison is possible at the higher temperatures. The reproducibility of the data may also be addressed, however, by observing how the 95% confidence limits on V_{max} depend on temperature. When normalized relative to the increased velocity, the spread in the data at 30°C, 0 V_i ($\pm$ 0.13 l/s) is approximately 20% less than at 10°C. The normalized confidence limits at 30°C and 1 mM V_i are approximately 20% greater than at the lower temperature. Similar observations are made for the remainder of the V_{max} data given in Fig. 8. Thus, using the relative magnitude of the statistical scatter in the data as a measure of data reproducibility, we conclude that we obtain no greater scatter at the higher temperatures than at the lower temperatures.

An additional concern was that our observed discontinuity in the effect of V_i on velocity at the higher temperatures might be an artifact induced by an inability of the experimental apparatus to reliably follow the shortening fiber at the higher velocities. Two observations argue against this possibility. First, we observe no change in V_{max} upon addition of 1 mM V_i at both 25 and 30°C. At the higher temperature, 30°C, we find that fibers shorten 30% faster than at 25°C (6.21 vs. 4.73 l/s). Thus, we initially observe the transition in behavior upon addition of V_i at velocities that are lower than the maximum velocity at which the apparatus is required to track in these experiments. Second, as is evident from Fig. 8, our observed Q_{10} for the increase in V_{max} with temperature over the range 5–30°C is approximately 2. This value for the Q_{10} is comparable to that observed for living fibers (reviewed in Woledge et al., 1985). Direct comparison with living rabbit psoas muscle at high temperatures is impossible. However, extrapolating our value for V_{max} to the physiological temperature of 39°C, we obtain a value for V_{max} of 11.4 l/s. This is close to the value of approximately 12 l/s expected for a 2- to 4-kg mammal based upon scaling with respect to size (see Chapter 9, McMahon, 1984, for discussion). Thus,

our agreement with data from living fibers provides additional confidence in our ability to accurately determine velocity in a shortening fiber at high temperatures.

The properties of vanadate

We conclude above that our protocols for obtaining the data have not introduced an artifact that could explain the observed effects of V_i on fiber mechanics. Are the effects due to other properties of V_i? V_i is added to the experimental buffers via dilution to pH 7 of a V_i stock that is maintained at pH 10. The high pH inhibits the formation of V_i oligomers. Upon reduction of the pH to 7, with $[V_i]$ in even the micromolar range, oligomer formation occurs (reviewed in Chasteen, 1983). This raises the possibility that the effects we see result from a polymerized species. Two points argue against this possibility, however. First, we observe that P_o is reduced to the same proportional levels upon addition of 1 mM V_i at 5, 15, and 25°C. We obtain tension levels of $0.11 \pm 0.02, n = 6$; $0.08 \pm 0.01, n = 3$; $0.10 \pm 0.02, n = 8$ (mean $\pm$ SE, number of observations) of the control values in the absence of added V_i, respectively. Thus, an explanation of our velocity results in terms of a polymerized species would require that the putative species bind and affect P_o equivalently at all temperatures, while having a completely different temperature effect on V_{max}. Second, oligomer formation is slow on a time scale relative to that required for collecting data from a single mounted fiber (Chasteen, 1983). Oligomer formation would be expected to be more rapid at high, not low temperatures, requiring that oligomer formation and velocity inhibition be inversely correlated. Hence, although we cannot completely eliminate the possibility that oligomer formation is involved, we are unaware of any molecular mechanism by which it might be accomplished.

The effects of cross-bridge number

Fiber stiffness decreases with increasing $[V_i]$ (Chase et al., 1993; Wilson et al., 1991). If this results from decreased cross-bridge attachment, the velocity of shortening in high $[V_i]$ can also be inhibited due to the small number of myosins operating on each filament. Such inhibition is seen when individual actin filaments are translated in vitro . This inhibition results from the fact that an individual myosin cross-bridge interacts with actin for only a limited part of its chemomechanical cycle (Pate and Cooke, 1991). For example, when an actin filament interacts with only a single myosin molecule, the in vitro assay translation velocity is decreased by a factor of 20 when compared with translation resulting from a large number of myosins (Uyeda et al., 1991). These data suggest that at maximal shortening velocity, the myosin head spends only 5% of its cycle attached and generating force. However, neither of these mechanisms appear to be working in fibers at high temperatures because V_{max} remained unchanged even at very low maximally Ca^{2+}-activated force levels induced by high $[V_i]$. This result is obtained despite the fact that at the lower tensions each actin filament would be interacting, on average, with one or fewer myosin heads. There are 300 myosin heads per thick filament. Assuming that each head spends 5% of its cycle attached to actin, at force levels of around 10% of maximal, on average, only 1.5 myosin heads per thick filament per half sarcomere would be generating force, and 0.75 myosin heads would be attached to a thin filament. However, this type of transition is not expected to change with temperature, nor could it produce the sharp transition region shown in Fig. 5. The lack of inhibition observed in skinned fibers at high $[V_i]$ at temperatures above 25°C shows that the filaments do not cease movement when the myosin heads are detached. For this to occur both the myosin filaments and the actin filaments of intact myofibrils must be tightly coupled in parallel. The data of Uyeda et al. (1991) show that 50–100 myosin heads are required to obtain maximum velocity. Thus, filaments that are far removed from each other in the myofibril must be coupled in parallel to within the distance of a cross-bridge powerstroke.

The role of cross-bridges weakly bound to actin

With increasing $[V_i]$, cross-bridge populations are thought to be shifted from strongly bound, force-producing states to $M \cdot ADP \cdot V_i$ states that bind only weakly to actin. These weakly binding cross-bridges could provide a drag that would slow velocity as we observe at lower temperatures. The ability of weakly bound cross-bridges to provide a drag on filament sliding is a property not generally attributed to cross-bridges in this state. Nonetheless, data consistent with this ability currently exist. Unphosphorylated smooth muscle myosin, a classic weakly binding cross-bridge, slows the filament translation velocity generated by fast skeletal muscle myosin in in vitro motility assays (Sellers et al., 1985; Warshaw et al., 1990). Chemical modification of skeletal muscle myosin with pPDM results in an analog of the weakly bound state (Chalovich et al., 1983). A similar slowing of in vitro translation velocity was observed when pPDM-modified myosin was mixed with native skeletal myosin (Warshaw et al., 1990). Weakly binding cross-bridges have also been invoked to explain the proportional decrease in isometric tension and shortening velocity seen for chemically skinned muscle fibers activated in a series of substrate analogs (Wang et al., 1993).

Our results suggest that several weakly bound cross-bridge states exist, with populations that depend dramatically on both the ligand attached to myosin and on temperature. In the presence of V_i, an $A \cdot M \cdot ADP \cdot V_i$ cross-bridge exerts a drag at low temperatures but not at high temperatures, with a very sharp transition between these two regimes. In contrast, at physiological [MgATP], no inhibition of shortening velocity is observed in the presence of high levels of P_i at either low temperatures, 10–13°C, (Chase and Kushmerick, 1988; Cooke et al., 1988) or at high temperatures (30°C) when the temperature jump protocols described here are used (Pate, Bhimani, and Cooke, unpublished observations). This suggests that weakly bound $A \cdot M \cdot ADP \cdot P_i$ cross-bridges do not

exert a drag on filament sliding velocity at any temperature. It is possible that the A·M·ADP·V_i cross-bridge mimics a transition state of the A·M·ADP·P_i cross-bridge that is not highly populated in the presence of P_i.

Cooperative transitions

The transition between the two distinct regions of V_i-induced behavior (Fig. 5) is extremely sharp, occurring over <5°C. Such a sharp transition would seem unlikely to result from a simple, continuous variation in kinetic parameters. Such behavior is generally seen only in highly cooperative reactions involving many subunits. In the muscle system, there are two obvious structures composed of a number of protein subunits that are capable of exhibiting cooperative behavior: the thick filament and the thin filament.

The thick filament provides one possibility of a cooperative interaction. At low temperatures, the myosin heads of relaxed fibers are disordered and spread from the thick filament where they might be expected to interact more easily with the thin filaments. At high temperatures, myosin heads assume an ordered helical array around the core of the thick filament, where they might be expected not to interact with actin thin filaments (Wray, 1987). The transition between ordered and disordered structures involves a large number of myosin heads, and thus could possibly involve cooperative interactions between the heads. The effect of temperature on this ordering is unknown. However, phosphorylation of the myosin head has been shown to disorder the myosin heads with respect to the thick filament even at high temperatures (Levine et al., 1991). Hence, we repeated the protocols for Fig. 5 at 20 and 25°C using fibers in which the myosin heads had been phosphorylated. We found V_i to inhibit V_{max} at 20°C and to leave V_{max} unaffected at 25°C. Thus, the limited data available indicate that transitions between ordered and disordered myosin heads are not the source of the sharp transition observed in Fig. 5. It is possible, however, that other transitions within the thick filament play a role.

The second possibility for cooperative protein interactions involves the thin filament. It has been shown that cooperativity exists within the thin filament, mediated via both calcium and the presence of active cross-bridges (Greene and Eisenberg, 1980; Zot, 1987). Our results could be explained if the interaction of a M·ADP·V_i complex with the regulated actin filament allows it to bind to and to exert a drag on thin filament motion at low temperature, but not at high temperatures. This hypothesis could be explored by altering the energetics of protein interactions within the thin filament.

SUMMARY

In summary, we have developed new experimental protocols that allow observations of isotonic shortening velocities to be made on skinned fibers contracting at temperatures approaching physiological levels. Employing these protocols, we find a surprising temperature dependence of the V_i-inhibition of shortening velocity. The dramatic loss of the ability of V_i to inhibit shortening velocity over a temperature range of <5°C would appear to require cooperative phenomena to be present in the filament array.

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REFERENCES

Altringham, J. D., and I. A. Johnston. 1985. Effects of phosphate on the contractile properties of fast and slow muscles from an antarctic fish. *J. Physiol.* 368:491–500.

Bershitsky, S. Y., and A. K. Tsaturyan. 1992. Tension responses to joule temperature jump in skinned rabbit muscle fibres. *J. Physiol.* 447:425–448.

Brozovich, F. V., L. D. Yates, and A. M. Gordon. 1988. Muscle force and stiffness during activation and relaxation. Implications for the actomyosin ATPase. *J. Gen. Physiol.* 91:399–420.

Chalovich, J. M., L. E. Greene, and E. Eisenberg. 1983. Cross-linked myosin subfragment 1: a stable analogue of the subfragment-1 ATP complex. *Proc. Natl. Acad. Sci. USA.* 80:4909–4913.

Chase, P. B., and M. J. Kushmerick. 1988. Effects of pH on contraction of rabbit fast and slow skeletal muscle fibers. *Biophys. J.* 53:935–946.

Chase, P. B., D. A. Martyn, M. J. Kushmerick, and A. M. Gordon. 1993. Effects of inorganic phosphate analogs on stiffness and unloaded shortening of skinned fibres from rabbit. *J. Physiol.* 460:231–246.

Chasteen, N. D. 1983. The biochemistry of vanadium. *Structure and Bonding.* 53:107–127.

Close, R. I. 1972. Dynamic properties of mammalian skeletal muscles. *Physiol. Rev.* 52:129–197.

Cooke, R. 1986. The mechanism of muscle contraction. *CRC Crit. Rev. Biochem.* 21:53–118.

Cooke, R., and W. Bialek. 1979. Contraction of glycerinated muscle fibers as a function of MgATP concentration. *Biophys. J.* 28:244–258.

Cooke, R., and E. Pate. 1985. The effects of ADP and phosphate on the contraction of muscle fibers. *Biophys. J.* 48:789–798.

Cooke, R., K. Franks, G. B. Luciani, and E. Pate. 1988. The inhibition of rabbit skeletal muscle contraction by hydrogen ions and phosphate. *J. Physiol.* 395:77–97.

Dantzig, J. A., and Y. E. Goldman. 1985. Suppression of muscle contraction by vanadate: mechanical and ligand binding studies on glycerol-extracted rabbit fibres. *J. Gen. Physiol.* 86:305–327.

Dantzig, J. A., Y. E. Goldman, N. C. Millar, J. Lacktis, and E. Homsher. 1992. Reversal of the cross-bridge force-generating transition by photogeneration of phosphate in rabbit psoas muscle fibers. *J. Physiol.* 451: 247–278.

Davis, J. S., and W. F. Harrington. 1993. A single order-disorder transition generates tension during the Huxley-Simmons phase 2 in muscle. *Biophys. J.* 65:1886–1898.

Goldman, Y. E. 1987. Kinetics of the actomyosin ATPase in muscle fibres. *Ann. Rev. Physiol.* 49:637–654.

Goldman, Y. E., J. A. McCray, and K. W. Ranatunga. 1987. Transient tension changes initiated by laser temperature jump in rabbit psoas fibres. *J. Physiol.* 392:71–95.

Goodno, C. C. 1982. Myosin active site trapping with vanadate ion. *Methods Enzymol.* 85:116–123.

Godt, R. E., and B. D. Lindley. 1982. Influence of temperature upon contractile activation and isometric force production in mechanically skinned muscle fibers of the frog. *J. Gen. Physiol.* 80:279–297.

Greene, L. E., and E. Eisenberg. 1980. Cooperative binding of myosin subfragment-1 to the actin-troponin-tropomyosin complex. *Proc. Natl. Acad. Sci. USA.* 77:2616–2620.

Hibberd, M. A., J. A. Dantzig, D. R. Trentham, and Y. E. Goldman. 1985. Phosphate release and force generation in skeletal muscle fibers. *Science.* 228:1317–1319.

Hibberd, M. A., and D. R. Trentham. 1986. Relationships between chemical and mechanical events during muscular contraction. *Ann. Rev. Biophys. Biophys. Chem.* 15:119–161.

Hill, A. V. 1938. The heat of shortening and the dynamic constants of muscle. *Proc. Royal Soc. Lond. B.* 126:136–195.

Johnston, I. A., and B. D. Sidell. 1984. Differences in temperature dependence of muscle contractile properties and myofibrillar ATPase Activity in a cold temperature fish. *J. Exp. Biol.* 111:179–189.

Lannergren, J., P. Lindblom, and B. Johansson. 1982. Contractile properties of two varieties of twitch fibres in *Xenopus laevis*. *Acta Physiol. Scand.* 114:523–535.

Levine, R. J. C., P. D. Chantler, R. W. Kensler, and J. L. Woodhead. 1991. Effects of phosphorylation by myosin light chain kinase on the structure of *Limulus* thick filaments. *J. Cell. Biol.* 113:563–572.

McMahon, T. A. 1984. Muscles, Reflexes, and Locomotion. Princeton University Press, Princeton, NJ. Chapter 9.

Martyn, D. A., and A. M. Gordon. 1992. Force and stiffness in glycerinated rabbit psoas fibers: effects of calcium and elevated phosphate. *J. Gen. Physiol.* 99:795–816.

Millar, N. C., and E. Homsher. 1990. The effect of phosphate and calcium on force generation in glycerinated rabbit skeletal muscle fibers. A steady state and transient kinetic study. *J. Biol. Chem.* 265:20234–20240.

Moerland, T. S., and B. D. Sidell. 1986. Contractile responses to temperature in the locomotory musculature of striped bass *Morone saxatilis*. *J. Exp. Zool.* 240:25–33.

Pate, E., and R. Cooke. 1989. Addition of phosphate to active muscle fibers probes actomyosin states within the powerstroke. *Pflügers Arch.* 414: 73–81.

Pate, E., and R. Cooke. 1991. Simulation of stochastic processes in motile cross-bridge systems. *J. Muscle Res. Cell Motil.* 12:376–393.

Penningroth, S. M. 1986. Erythro-9-[3-(2-hydroxynonyl)]adenine and vanadate as probes for microtubule-based cytoskeletal mechanochemistry. *Methods Enzymol.* 134:477–487.

Ranatunga, K. W. 1984. The force-velocity relation of rat fast- and slow-twitch muscles examined at different temperatures. *J. Physiol.* 351:517–529.

Rayment, I., W. R. Rypniewski, K. Schmidt-Base, R. Smith, D. R. Tomchick, M. M. Benning, D. A. Winkelmann, G. Wesenberg, and H. M. Holden. 1993a. Three-dimensional structure of myosin subfragment-1: a molecular motor. *Science.* 261:50–58.

Rayment, I., H. M. Holden, M. Whittaker, C. B. Yohn, M. Lorenz, K. C. Holmes, and R. A. Milligan. 1993b. Structure of the actin-myosin complex and its implications for muscle contraction. *Science.* 261:58–65.

Rubinson, K. A. 1981. Concerning the form of biochemically active vanadium. *Proc. Royal Soc. Lond. B.* 212:65–84.

Sellers, J. R., J. A. Spudich, and M. P. Sheetz. 1985. Light chain phosphorylation regulates the movement of smooth muscle myosin on actin filaments. *J. Cell. Biol.* 101:1897–1902.

Stein, R. B., T. Gordon, and J. Shriver. 1982. Temperature dependence of mammalian muscle contractions and ATPase activities. *Biophys. J.* 40: 97–107.

Uyeda, T. Q. P., S. J. Kron, and J. A. Spudich. 1991. The myosin step size: estimation from slow sliding movement of actin over low densities of heavy meromyosin. *J. Mol. Biol.* 214:699–710.

Walker, J. W., and R. L. Moss. 1992. Effects of Ca^{2+} on the kinetics of phosphate release in skeletal muscle. *J. Biol. Chem.* 267:2459–2466.

Wang, D., E. Pate, R. Cooke, and R. G. Yount. 1993. Synthesis of non-nucleotide ATP analogues and characterization of their chemomechanical interaction with muscle fibers. *J. Muscle Res. Cell Motil.* 14:487–497.

Warshaw, D. M., J. M. Desrosiers, S. S. Work, and K. M. Trybus. 1990. Smooth muscle myosin cross-bridge interactions modulate actin sliding velocity in vitro. *J. Cell. Biol.* 111:453–463.

Wilson, G., J. Paige, and R. Cooke. 1990. Trapping spin-labeled ADP on myosin heads using vanadate. *Biophys. J.* 57:330a.

Wilson, G., S. Shull, and R. Cooke. 1991. Myosin heads function independently during force generation. *Biophys. J.* 59:51a. (Abstr.)

Woledge, R. C., N. A. Curtin, and E. Homsher. 1985. Energetic Aspects of Muscle Contraction. Academic Press, London. Chapter 2.

Wray, J. S. 1987. Structure of relaxed myosin filaments in relation to nucleotide state in vertebrate skeletal muscle. *J. Muscle Res. Cell Motil.* 8:62.

Zot, A. S. 1987. Structural aspects of troponin-tropomyosin regulation of skeletal muscle contraction. *Ann. Rev. Biophys.* 16:535–559.